ABSTRACTS (PH D THESIS)

Lipid secretion mechanism in *Lithospermum erythrorhizon* using shikonin as a model of hydrophobic metabolites

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Introduction

Plants synthesize a large number of metabolites for adaptation to their environmental stresses. Among them, lipophilic secondary metabolites often accumulate in extracellular spaces and function as barrier to biotic and abiotic stresses. For instance, in Laminaceae plants, monoterpenes are produced by secretory cells of glandular trichomes and accumulate in the epicuticular cavity [1]. Furanocoumarins are biosynthesized in the peels (flavedo) of citrus species and accumulate specifically in oil cavities [2]. Moreover, all land plants secrete cutin, lipophilic polymers, to protect the plant tissues from external aggressions and passive water loss [3]. However, it is largely unknown how these lipophilic metabolites are secreted and accumulated in extracellular spaces. To investigate the transport of plant lipophilic secondary metabolites, I have used in this study shikonin derivatives as a model compound of lipophilic endogenous substances. This red pigment is produced in the root epidermal cells of *Lithospermum erythrorhizon* and secreted from these cells into the apoplastic spaces. The shikonin production system is an excellent model system because of three major benefits; 1) shikonin is a visible pigment, 2) production of shikonin is clearly regulated as 0 to 100% manner by medium composition and illumination, 3) there are culture system of shikonin-producing cells and hairy root cultures. These properties may lead to greater understandings of the intracellular trafficking of lipophilic metabolites [4]. This Ph.D thesis provides several biochemical evidences relevant for the excretion of lipophilic metabolites from plant cells.

Results

Establishment of *L. erythrorhizon* transformation using domestic *Rhizobium rhizogenes* strain A13

An effective transformation method is required to conduct the researches mentioned above. However, standard *L. erythrorhizon* transformation methods have not yet been established. To overcome this problem, I established a methodology of effective stable transformation of *L. etyrhrorhizon* using a domestic *Rhizobium rhizogenes* strain A13 as a carrier (Fig. 1). This protocol achieved high transformation efficiency (50 - 70%) [5]. Furthermore, an inducible transcriptional regulation system was introduced into the hairy root culture to prevent a large clonal variation that obstructs the evaluation of gene functions of interests. In conclusion, our protocols allowed *in-planta* functional analysis of candidate genes from *L. erythrorhizon*, which may contribute to understanding the molecular mechanisms of shikonin biosynthesis and secretion.

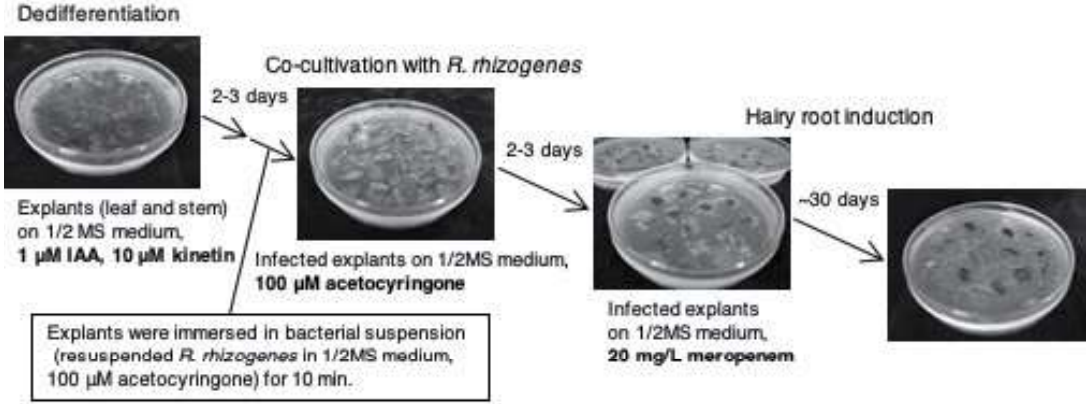

Figure 1. Scheme of the transformation method of *L. erythrorhizon.* (sited from [5])

ABSTRACTS (PH D THESIS)

Analysis of subcellular events accompanied with shikonin production using hairy roots

The biosynthesis of shikonin derivatives has been intensively studied. A key regulatory reaction in its biosynthetic pathway is the conjugation of a geranyl moiety to *p*-hydroxybenzoic acid, yielding *m*-geranyl-*p*-hydroxybenzoic acid. This reaction is catalyzed by a membrane-bound prenyltransferase, LePGT [6]. To identify the organelle involved in shikonin synthesis, I analyzed the subcellular localization of this enzyme. It was shown that the LePGT localized at endoplasmic reticulum (ER). Moreover, in electron microscopic analysis, highly developed ER was observed in shikonin-production epidermal cells. Next, I searched for inhibitors of shikonin secretion in hairy root cultures, and identified two compounds, cytochalasin D, an inhibitor of actin filament polymerization, and brefeldin A, an inhibitor of the adenosine diphosphate (ADP)-ribosylation factor/guanine nucleotide exchange factor (ARF/GEF) protein system, which are often used in membrane traffic studies. These results suggested that the secretion of shikonin derivatives into the apoplast utilizes the pathways common to the ARF/GEF system and actin filament polymerization, at least in part [7].

Identification of the matrix lipid involved in shikonin transport mechanism

As shikonin is a very lipophilic substance, it is rapidly crystallized in water and cytosol as well as in the medium, while it is accumulated as red droplet/granules at the surface of the living cells and root tissues of *L. erythrorhizon*. It is therefore presumed that a matrix lipid plays an important role in solubilizing and secreting shikonin safely out of the cells. In an attempt to identify the lipids, we analyzed secreted shikonin derivatives by electron microscopy and chemical analysis of secreted lipids, suggesting that shikonin derivatives form oil droplet structures with matrix lipids and polar lipids.

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References

[1] B Markus Lange and Rodney Croteau. (1999) *Current Opinion in Plant Biotechnology*, 2: 139-144.
[2] Sian Sie Voo, Howard D. Grims, B Markus Lange. (2012) *Plant physiology,* 159:81-94.
[3] Christiane Nawrath, Lukas Schreiber, Rochus Benni Franke, Niko Geldner, Jose J Reina-Pinto, Ljerka Kunst. (2013) *Arabidopsis Book,* 11: e0167.
[4] Kazufumi Yazaki (2017) *Plant Biotechnol. (Tokyo)*, 34: 131-142.
[5] Kanade Tatsumi, Takuji Ichino, Noboru Onishi, Koichiro Shimomura, Kazufumi Yazaki. (2020) *Plant Biotechnol. (Tokyo)*, 37: 39-46.
[6] Ohara K, Muroya A, Fukushima N, Yazaki K (2009) *Biochem J*, 421: 231–241.
[7] Kanade Tatsumi, Mariko Yano, Kenta Kaminade, Akifumi Sugiyama, Mayuko Sato, Kiminori Toyooka, Takashi Aoyama, Fumihiko Sato, Kazufumi Yazaki (2016) *Front. Plant Sci.,* 7: 1066.